JOURNAL OF BACTERIOLOGY, Sept. 1985, p. 1114–1119
0021-9193/85/091114-06$02.00/0

Vol. 163, No. 3

Characterization of the Diphtheria *tox* Transcript in *Corynebacterium diphtheriae* and *Escherichia coli*

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Transcription of the *tox* gene in lysogenic *Corynebacterium diphtheriae* strains C7(β *tox*$^+$), C7(γ *tox*) and the hypertoxigenic PW8 (ω *tox*$^+$) was analyzed and compared with transcription of the *C. diphtheriae tox* gene in the recombinant strain *Escherichia coli*(pDT201). In all cases S1 nuclease mapping localized the 5′ terminus of the *tox* mRNA to a site 8 or 9 base pairs (bp) downstream of a region similar to the −10 consensus sequence of *E. coli* promoters. In *C. diphtheriae* the *tox* transcript was observed only in strains that were grown under iron-limiting conditions; in the presence of excess iron, transcription beyond bp 38 of the *tox* coding region was not observed. In contrast, in *E. coli*(pDT201) *tox* was expressed at equivalent levels in both iron-depleted and iron-supplemented media. The DNA insertion in the *tox* gene of the nontoxigenic corynephage γ was found to occur at bp 54 of the *tox* coding region. The insertion event resulted in the duplication of a 7-bp target sequence, and the ends of the insert were found to constitute an imperfect inverted repeat of approximately 26 bp. Transcription from the *tox* promoter in C7(γ *tox*) was found to initiate at the same nucleotides as in C7(β *tox*$^+$), PW8, and *E. coli*(pDT201) and remained sensitive to iron inhibition. These observations are discussed in relation to the mechanism of iron-mediated regulation of the *tox* gene.

The diphtheria toxin structural gene, *tox*, is carried by a number of closely related corynebacteriophages (14). The *tox*-encoding regions of the phages β *tox*$^+$, ω *tox*$^+$, and γ *tox* have been positioned on the restriction endonuclease digestion maps of their respective genomes (4, 7, 24, 32), and the nucleotide sequences of several β phage as well as the ω phage *tox* alleles have been determined (8, 11, 15, 33). The regulation of *tox* expression appears to be both independent of all other corynephage functions and mediated by a host-determined factor(s) (1, 10, 16). Maximal expression of *tox* occurs only during the decline phase of the bacterial growth cycle, when iron becomes the rate-limiting substrate (30).

The molecular cloning of an 831-base-pair (bp) *Sau*3A1 fragment of the β phage genome encoding the *tox* promoter, signal sequence, and all of fragment A of the toxin in plasmid pDT201 in *Escherichia coli* results in the expression and export of a fragment A-related protein (17, 18). Nucleotide sequence analysis of the region upstream of the *tox* structural gene has revealed sequences similar to the consensus −35 and −10 regions of *E. coli* promoters (15, 33). We have recently shown that this region cloned in the appropriate orientation in the promoter probe vector pKO-1 had moderate levels of promoter activity in *E. coli* (18).

We have subsequently focused our studies on the *Corynebacterium diphtheriae tox* mRNA and in this report show that the 5′ termini of the *tox* transcripts extracted from *E. coli*(pDT201) and *C. diphtheriae* strains C7(β *tox*$^+$) and the hypertoxigenic PW8(ω *tox*$^+$) are identical. In addition, we have localized the DNA insertion in the γ phage *tox* allele to a site 54 bp downstream of the GTG initiation codon and have shown that this insertion does not appear to affect either the initiation of transcription or the iron-mediated regulation of the *tox* allele in C7(γ *tox*).

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MATERIALS AND METHODS

Bacterial strains. The *C. diphtheriae* and *E. coli* strains that were used in this study are listed in Table 1.

Media. *E. coli* strains were grown in Luria broth (LB; 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) containing 100 μg of ampicillin per ml. Low-iron LB was prepared by adding 2 ml of 50% $CaCl_2$ and 5 g of KH_2PO_4 per liter, adjusting the pH to 7.4, boiling, and filtration through Whatman no. 40 paper.

C. diphtheriae strains were grown in C-Y medium (20 g of yeast extract, 10 g of Casamino Acids, 10 ml of 10% L-tryptophan, 2 ml of 50% $CaCl_2$, and 5 g of KH_2PO_4 per liter). The pH was adjusted to 7.4, and the medium was boiled and filtered through Whatman no. 40 paper, and 2 ml of solution II (25) and 1 ml of solution III (25) were added before sterilization. Sterile 50% maltose–0.5% $CaCl_2$ (3 ml per 100 ml of C-Y medium) was added before use. High-iron C-Y medium contained 500 μM $FeSO_4$. Culture conditions for *tox* expression have been described previously (32).

Subcloning of the *tox* promoter and galactokinase assay. The promoter probe vector pKO-1 was used for the subcloning of DNA fragments from pDT201, which carries the putative diphtheria *tox* promoter (22). Restriction endonucleases and DNA ligase (New England Biolabs, Beverly, Mass.) were used according to the specifications of the manufacturer. Procedures for molecular cloning, transformation of *E. coli* K-12, and screening for recombinant strains have been described previously (17). *E. coli* N100 and derivative strains were grown in M9 medium supplemented with 1% fructose, 200 μg of Casamino Acids (Difco Laboratories) per ml and 10 μg of ampicillin per ml. Bacteria were grown to an A_{590} of 0.6 to 0.8 and were lysed and assayed for galactokinase activity as previously described (18, 22).

ADPRT assay. ADP-ribosyl transferase (ADPRT) activity was measured essentially as described by Gill and Pappenheimer (9), except that wheat germ elongation factor 2 was substituted for rabbit reticulocyte elongation factor 2 (6).

TABLE 1. Bacterial strains and plasmids used in this study

Strain and plasmid	Reference or source
C diphtheriae	
C7(β *tox*$^+$)	2
C7(γ *tox*)	12
PW8(ω *tox*$^+$)	32
E. coli	
71-18(pDT201)	17
N100	22
N100(pKO-1)	22
N100(pDT299)	18
N100(pDT251)	18
N100(pDT252)	This study
N100(pDT253)	This study

RNA extraction. RNA was purified from *E. coli* strains 71-18(pDT201) and 71-18(pUC8) by hot phenol extraction as described by Salser et al. (34) and modified by Palmiter (29). Cultures were grown in 5-ml volumes to an A_{590} of approximately 0.5, poured over ice, and sedimented by centrifugation. Cells were suspended in 500 μl of 10 mM KCl–5 mM $MgCl_2$–10 mM Tris hydrochloride, pH 7.3, containing 300 μg of lysozyme per ml, and frozen at −70°C. The suspension was then thawed in the presence of 1% sodium dodecyl sulfate and incubated at 65°C until lysis occurred. Sodium acetate, pH 5.2, was added to 100 mM, and the lysate was extracted with an equal volume of phenol for 4 min at 65°C. Chloroform (500 μl) was added, and the mixture was centrifuged. The aqueous phase was removed and then extracted twice with 500 μl of chloroform. The concentration of sodium acetate was increased to 300 mM, and the RNA was precipitated by adding 2.5 volumes of absolute ethanol and chilling to −70°C.

C. diphtheriae strains to be used for RNA extraction were grown overnight in C-Y medium, harvested by centrifugation, and suspended in fresh medium to an A_{590} of approximately 1.5. Cultures were incubated at 34°C with shaking (240 rpm) until the A_{590} reached 4 to 5. The cells were then harvested, washed, suspended to an A_{590} of 9 in Chelex-100-treated medium, and incubated at 34°C with shaking. The production of diphtheria toxin was quantitated by rocket immunoelectrophoresis as previously described (27).

RNA was extracted from 500-μl samples of *C. diphtheriae* cultures essentially as described by Buck and Groman (4). The culture was poured over ice, and the cells were sedimented by centrifugation. The pellet was suspended in 50 mM sodium acetate–4% sodium dodecyl sulfate–50 mg of bentonite per ml–1 mg of yeast tRNA per ml. After incubation at 100°C for 1.5 min, an equal volume of chloroform-isoamyl alcohol (24:1) was added, and the suspension was vortexed for 1.5 min. Phenol (500 μl) was added, and the suspension was agitated for 30 min at 65°C. Following incubation, the mixture was centrifuged and the aqueous phase was removed. After an additional phenol extraction and two chloroform extractions, the RNA was precipitated with ethanol and stored at −70°C until used.

S1 nuclease mapping. The *C. diphtheriae tox* gene probe for S1 nuclease digestion experiments with RNA extracted from the C7(β *tox*$^+$) and PW8 *tox*$^+$ strains of *C. diphtheriae*, as well as *E. coli*(pDT201), was a subfragment of the 232-bp *Sau*96I (−177 to +55) fragment of pDT201. The DNA probe used for S1 nuclease mapping of the C7(γ *tox*) *tox*-related mRNA was derived from the 215-bp *Sau*96I-*Hin*P (−177 to +38) fragment. In both cases double-stranded DNA restriction endonuclease fragments of plasmid pDT201 were purified and end labeled with [γ-^{32}P]ATP as previously described (21). The fragments were then digested with *Hin*dIII to generate the probes indicated in the text. DNA strands were separated by electrophoresis in 5 to 8% polyacrylamide (acrylamide-bisacrylamide, 50:1) essentially as described by Maxam and Gilbert (21). Following electrophoresis, single strands were detected by autoradiography, eluted from the gel, and ethanol precipitated. The single-stranded DNA was then coprecipitated with 100 μg of RNA. The precipitate was dried and redissolved in 15 μl of high-salt buffer (750 mM NaCl, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 12 mM EDTA, pH 7.0) (13). The hybridization mixture was incubated at 68°C for 10 min and then at 55°C for 3 to 16 h. Hybridization was quenched with 200 μl of S1 nuclease buffer (250 mM NaCl, 30 mM sodium acetate, 1 mM $ZnSO_4$, 5% glycerol, pH 4.6) at 0°C. The hybrids were then digested with 1,000 U of S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 1 h at 37°C. After phenol-chloroform extraction, the digestion products were ethanol precipitated and electrophoresed on sequencing gels (0.4 mm by 40 cm) at 1,500 V for 1.5 to 3.0 h. ^{32}P-end-labeled DNA fragments used as the probe were cleaved at G and A and at C and T residues and were coelectrophoresed as molecular weight markers.

DNA sequencing. DNA fragments of the γ_c *tox* genome were sequenced by the method of Maxam and Gilbert (21) with modifications in the G+A reactions as described by Maniatis et al. (20).

RESULTS

5′ terminus of the *tox* transcript in *E. coli*. We have previously reported that a 232-bp segment of the *Hae*III fragment of pDT201 that carries the −35 and −10 promoter consensus sequences upstream of the *tox* structural gene had functional promoter activity when cloned in the promoter probe vector pKO-1 in *E. coli* (18). To define further the position of the sequences recognized as a promoter, smaller regions of the *Hae*III fragment were subcloned into the pKO-1 vector. A 174-bp *Hin*dIII- *Hae*III and a 67-bp *Alu*I segment of the *Hae*III insert were found to direct the expression of equivalent levels of galactokinase activity in derivative strains of *E. coli* N100 (Fig. 1, Table 2). These results indicate that the region recognized as a promoter in *E. coli* was located on the 67-bp *Alu*I fragment that extends from base −42 to base −109 upstream of the *tox* GTG initiation codon.

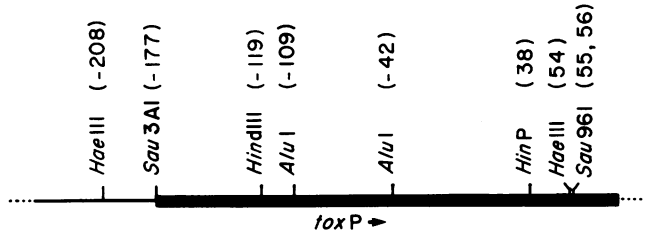

FIG. 1. Partial restriction endonuclease map of the portion of pDT201 (17) encoding the *C. diphtheriae tox* regulatory region and the *tox* signal sequence. Bold line, Cloned corynebacteriophage β_c *tox-45* sequences; thin line, pUC8 vector sequences. Numbers indicate distances (in base pairs) from the GTG *tox* translational start signal.

We selected a 174-bp *Sau*96I-*Hin*dIII fragment containing bases 55 to −119 of the *tox* gene to be used as a probe in S1 nuclease mapping experiments (Fig. 1). RNA was extracted from log-phase cultures of *E. coli* 71-18(pDT201) and then hybridized to single-stranded ^{32}P-labeled probe DNA. The hybrids were digested with S1 nuclease and electrophoresed on denaturing polyacrylamide gels adjacent to chemical cleavage ladders of the original probe. DNA fragments corresponding to positions −40 and −41 upstream of the *tox* structural gene were observed (Fig. 2, lane A). Identical results were obtained for RNA-DNA hybridization reactions which were incubated at 58 rather than 55°C and for S1 nuclease digestions of the hybrids at 15 and 45°C. In contrast, no protected DNA fragments were observed when yeast tRNA was substituted for the *E. coli*(pDT201) RNA preparation (Fig. 2, lane G) or when RNA from *E. coli*(pUC8) was used.

Since iron is known to inhibit *tox* expression in *C. diphtheriae* (30), the effect of iron on expression of the *tox* gene in *E. coli* was analyzed. *E. coli*(pDT201) was grown in LB medium that was either iron depleted or supplemented with 500 μM $FeSO_4$. Bacteria were lysed, and extracts were assayed for ADPRT activity. Neither treatment affected the level of ADPRT activity expressed by *E. coli* 71-18(pDT201) (data not shown).

5′ terminus of the *tox* transcript in *C. diphtheriae*. RNA was extracted from the lysogenic, toxinogenic *C. diphtheriae* strains C7(β *tox*$^+$) and PW8, which were expressing toxin in iron-depleted C-Y medium. These RNA preparations were then annealed to the *Sau*96I-*Hin*dIII probe as described above, and hybrids were digested with S1 nuclease. DNA fragments corresponding to positions −40 and −41 upstream of the *tox* structural gene were resolved (Fig. 2, lanes B and C). Identical results were obtained after hybridization at 58°C and S1 nuclease digestions at 15 and 45°C.

Both Murphy et al. (28) and Costa et al. (7) demonstrated that only RNA extracted from iron-limited cultures of *C. diphtheriae* would hybridize to β phage DNA. In contrast, RNA extracted from cells grown in the presence of excess iron did not detectably hybridize to β phage DNA. Since S1 nuclease mapping is a more sensitive means of transcript detection, we extracted RNA from C7(β *tox*$^+$) grown in the presence of 500 μM iron. RNA extracted from these cells failed to protect the ^{32}P-labeled DNA probe (Fig. 2, lane F).

Transcription of *tox* sequences in *C. diphtheriae* C7(γ *tox*). Comparison of the restriction endonuclease digestion maps of corynephages β *tox*$^+$ and γ *tox*, as well as heteroduplex analysis, demonstrated a DNA insertion of 1.2 to 1.75 kilobases in the γ phage genome (3, 5, 24). Furthermore, this insertion was estimated to be either in the *tox* regulatory region or early in the toxin structural gene (24). To localize the γ phage insertion further, fragments of the γ_c *tox* phage genome containing the junctions between the insert and the *tox* structural gene were cloned in the pUC8 vector in *E. coli*. The nucleotide base sequence of these subfragments was then determined by the method of Maxam and Gilbert (21) and compared with the sequences of the β and ω phage *tox* genes. The insertion in the γ *tox* phage genome began at base +54 in the *tox* structural gene (Fig. 3).

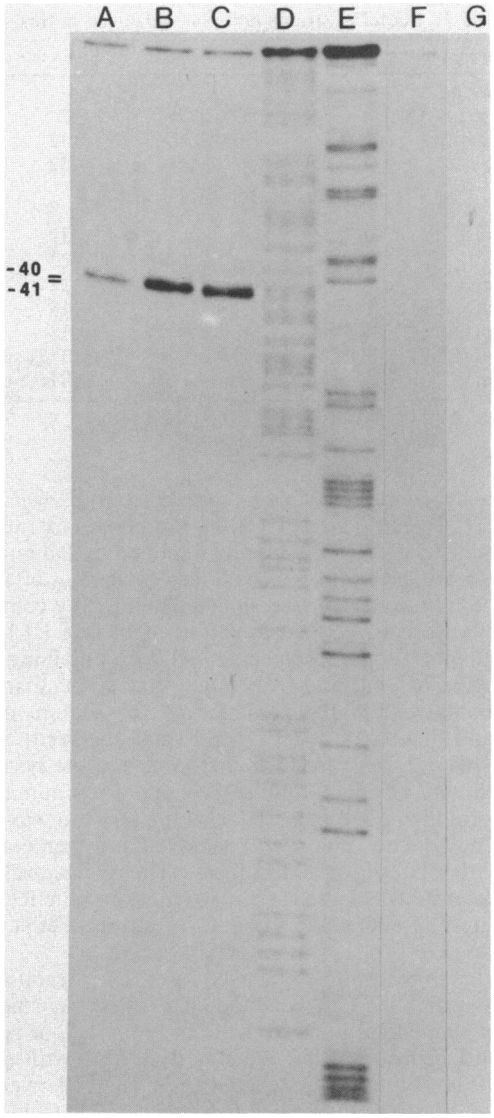

FIG. 2. Autoradiograph of a sequencing gel of S1 nuclease analysis of the *tox* transcript in *E. coli* and *C. diphtheriae*. Lanes A, B, C, F, and G, S1 nuclease digestion products obtained with RNA from (A) *E. coli* 71-18(pDT201), (B) *C. diphtheriae* C7(β *tox*$^+$) grown in low-iron conditions, (C) *C. diphtheriae* PW8 grown in low-iron conditions, (F) C7(β *tox*$^+$) grown in conditions of iron excess, and (G) yeast tRNA. Lanes D and E, G+A (D) and T+C (E) sequencing reactions of the probe. Arrow, Major S1 nuclease digestion products. Numbers indicate the corresponding site in the sequence upstream of the *tox* coding region.

TABLE 2. Galactokinase activity of *E. coli* N100 strains carrying the promoter probe vector pKO-1 and derivatives carrying the 262-bp *Hae*III fragment or subfragments of pDT201

E. coli strain	Restriction endonuclease fragment (bp)	Galactokinase activity (U/ml)[a]
N100		0
N100(pKO-1)		0.81
N100(pDT299)[b]	*Hae*III (262)	26
N100(pDT251)[c]	*Hae*III (262)	209
N100(pDT253)	*Hin*dIII-*Hae*III (174)	200
N100(pDT252)	*Alu*I (67)	225

[a] Galactokinase activity was assayed in *E. coli* N100 derivatives. Enzymatic activity is expressed as units per milliliter of culture at an A_{590} of 1.0.

[b] pDT299 carries the *C. diphtheriae tox* promoter region in pKO-1 in the opposite orientation with respect to *tox*.

[c] pDT251 carries the *C. diphtheriae tox* promoter region in pKO-1 in the same orientation with respect to *tox*.

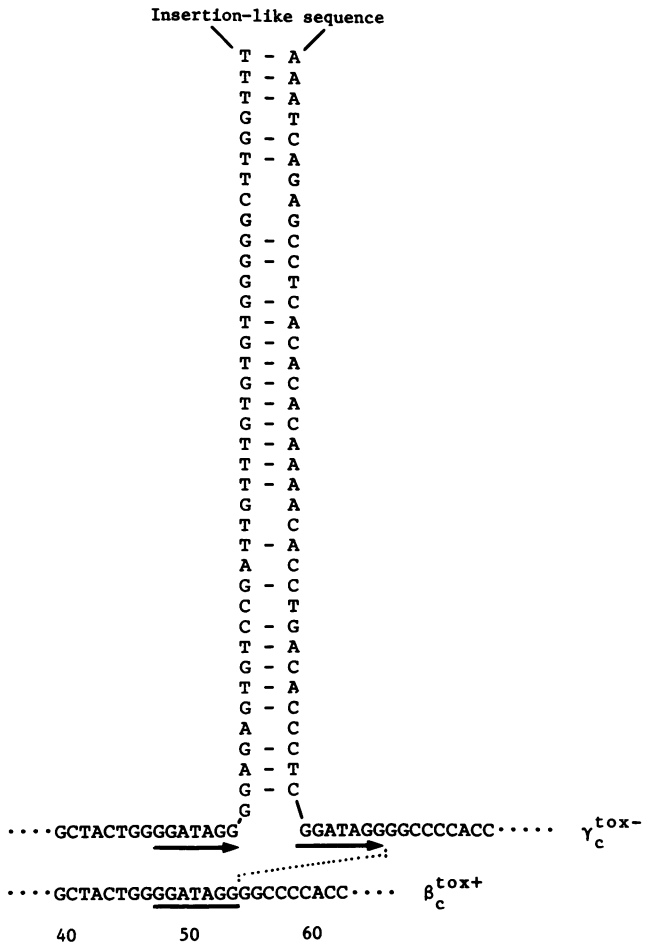

FIG. 3. Nucleotide base sequence of the ends of the insertion in the *tox* gene of γ_c. The upper line represents the γ_c sequence, with the 7-bp direct repeat underlined with arrows and the potential base pairing in the inverted repeats indicated by a dotted line. The lower line represents the corresponding β_c *tox* sequence in which the insertion occurred. Numbers indicate the distance (in base pairs) from the GTG initiation signal.

The nucleotide base sequence analysis of the junctions between the γ phage *tox* allele and the DNA insertion revealed features characteristic of an insertion sequence. In particular, there was an imperfect inverted repeat of approximately 26 bp flanked by a 7-bp repeat of *tox* sequences at the insertion site (Fig. 3). Except for the insertion, there were no differences in the nucleotide sequence of the γ *tox* allele from those of the β and ω *tox* alleles in this region.

DNA sequence analysis of the *tox* gene indicated the presence of a *Hin*P restriction site upstream of the insertion sequence. As a result, a DNA fragment from the *Hin*dIII (−119) to the *Hin*P (+38) sites was used as a probe for S1 nuclease mapping of *tox*-related transcripts in C7(γ *tox*) (Fig. 1). *C. diphtheriae* strains C7(β *tox*$^+$) and C7(γ *tox*) were grown under conditions of iron excess or iron starvation, RNA was extracted and hybridized to the *Hin*dIII-*Hin*P probe, and the hybrids were digested with S1 nuclease. RNA from both strains grown under iron-limiting conditions yielded protected fragments of identical size (Fig. 4, lanes A and B). In addition, equivalent amounts of RNA from the two strains gave bands of comparable intensity. As anticipated, RNA extracted from either C7(β *tox*$^+$) or C7(γ *tox*) grown in the presence of excess iron failed to protect the DNA probe from S1 nuclease digestion (Fig. 4, lanes E and F). These results suggest that transcription in cells grown in excess iron does not proceed beyond bp 38 of the *tox* gene.

DISCUSSION

The expression of cloned diphtheria toxin structural gene (*tox*) fragments in *E. coli* has been shown to be independent of the orientation of the DNA insert within the vector (15, 17, 35). These observations suggested that sequences within the regulatory region of the *tox* gene were recognized as signals for the initiation of transcription in *E. coli*. We have previously demonstrated that a 232-bp DNA fragment con-

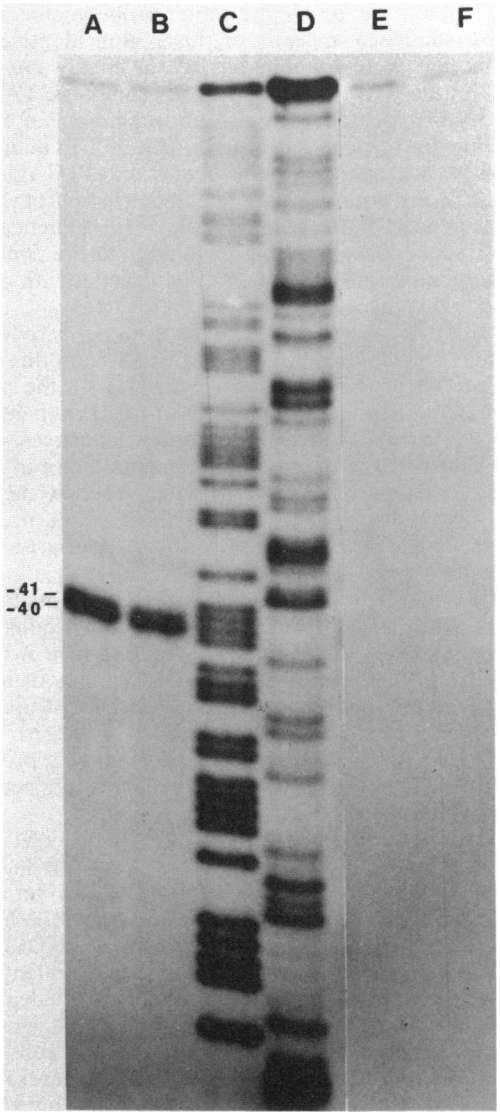

FIG. 4. Autoradiograph of a sequencing gel of S1 nuclease analysis of the *tox*-related transcript in *C. diphtheriae* C7(γ *tox*). Lanes A, B, E, F, and G show the S1 nuclease digestion products obtained with RNA from (A) C7(β *tox*$^+$) grown in low-iron conditions, (B) C7(γ *tox*) grown in low-iron conditions, (E) C7(β *tox*$^+$) grown in high-iron conditions, (F) C7(γ *tox*) grown in conditions of iron excess, and (G) yeast tRNA. Lanes C and D, G+A (C) and T+C (D) sequencing reactions of the probes. Numbers indicate the positions of the corresponding nucleotides upstream of the *tox* coding region.

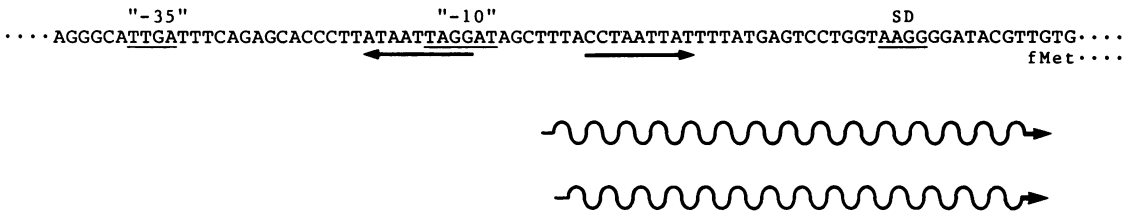

FIG. 5. Features of the regulatory region of the *tox* gene. The putative *tox* promoter sequences are indicated as −10 and −35 and are underlined. The 5′ ends of the *tox* mRNA are designated by wavy arrows and initiate at positions −40 and −41 from the translational start signal. SD, Putative ribosome-binding site. Straight arrows indicate the inverted repeat in the *tox* regulatory region.

taining the putative *tox* promoter sequences had moderate levels of promoter activity (18) when cloned in the appropriate orientation in the pKO-1 promoter probe vector. Nucleotide base sequence analysis of this region identified two sequences that were homologous to the highly conserved TATAAT at the −10 position of *E. coli* promoters: TATAAT and TAGGAT, located 54 and 48 bp upstream of the *tox* structural gene, respectively (Fig. 5) (15, 33). In addition, a TTGA sequence similar to the conserved TTGACA −35 region of *E. coli* promoters was detected −74 bp from the *tox* structural gene. Of the two possible −10 sequences, the TAGGAT sequence was less homologous to the consensus −10 sequence but had more favorable spacing with respect to the −35 sequence.

To localize the site(s) for functional promoter activity in *E. coli* further, we subcloned DNA fragments from this region into the pKO-1 promoter probe vector (18). In the present report we demonstrate that a 67-bp *Alu*I fragment containing the putative *tox* −10 and −35 promoter sequences was as active in directing the expression of galactokinase in *E. coli* as were the larger DNA inserts which carried these sequences. These studies suggest that most of the promoter activity of the *tox* regulatory region in *E. coli* arises from sequences contained within the *Alu*I fragment.

We performed S1 nuclease mapping experiments to characterize the *tox* transcript made in toxigenic strains of *C. diphtheriae* and in *E. coli* expressing the fragment A-related polypeptide. In *E. coli*(pDT201) the 5′ terminus of the *tox* mRNA was found to initiate at bases −40 and −41 upstream from the GTG translational start signal of the *tox* gene. Whether TATAAT or TAGGAT or both confer *tox* promoter activity in *E. coli* is not known; this question could be resolved by site-directed mutagenesis.

C. diphtheriae tox transcripts were detected by S1 nuclease mapping in *C. diphtheriae* C7(β *tox*$^+$) and PW8(ω *tox*$^+$) cells grown under iron-limiting conditions. The 5′ termini of the *tox* mRNAs in both *C. diphtheriae* and *E. coli* were found to be the same. Similar results for *E. coli*(pTD134) and *C. diphtheriae* C7(β *tox-228*) have been independently obtained by Kaczorek and co-workers by both S1 nuclease and primer extension analyses (15a). These results suggest that the corynebacterial RNA polymerase present in cells growing under iron-limiting conditions recognizes features similar to those recognized by the *E. coli* polymerase. The −10 and −35 sequences characterized in *E. coli* have been found to be conserved in promoters of gram-positive organisms, such as the *Bacillus subtilis* promoters which are recognized by RNA polymerase containing σ^{55} (19) and the β-lactamase promoter in *Staphylococcus aureus* (23). Information relating to this hypothesis, such as other characterized corynebacterial or corynephage promoters or *tox* promoter mutations, is not yet available.

We did not detect the presence of *tox* transcripts proceeding beyond bp 38 in *C. diphtheriae* strains grown in the presence of excess iron. These results confirm and extend those of earlier studies in which ^{32}P-labeled RNA was used as a probe in Southern hybridization experiments (4, 7). The results suggest that *tox* gene expression is regulated by iron at the level of transcription, possibly at the initiation of transcription. The alternative possibility, that transcription initiates at but does not extend beyond bp 38 in the presence of iron, cannot be ruled out by the experiments reported here. In *E. coli*(pDT201), the expression of the fragment A-related polypeptide is apparently not regulated by iron since we have found that equivalent levels of ADPRT were produced in cultures grown in either iron-depleted or iron-supplemented medium.

In 1976, Murphy and Bacha (26) proposed a model for *tox* regulation in which the inhibitory effect of iron was mediated through an aporepressor which acted as a negative controling factor. While the genetic evidence so far obtained does not offer a compelling argument for either positive or negative control of *tox* expression, the finding that the 67-bp *Alu*I insert had moderate levels of promoter activity in *E. coli* is consistent with the idea of a corynebacterial factor which represses *tox* expression in *C. diphtheriae* growing in the presence of high concentrations of iron. Although the mechanism of *tox* regulation is not understood, the simplest model continues to be one of negative control.

We have shown that the *tox* gene of corynephage γ *tox* contains an insertion that interrupts the *tox* gene at bp +54. This site is within the region of the *tox* structural gene encoding the signal peptide. The insertion has properties in common with procaryotic insertion sequences: (i) the ends constitute an imperfect 26-bp inverted repeat, and (ii) there is a 7-bp duplication of *tox* sequences at the site of insertion. Similar results have been independently obtained by Rappuoli and Ratti (personal communication). In addition, both Buck and Groman (3) and Rappuoli (personal communication) have found multiple fragments in restriction digests of *C. diphtheriae* chromosomal DNA which hybridize with the insertion. These results suggest that the insertion in the γ phage *tox* gene is, in fact, an insertion sequence.

The *tox* gene of corynephage γ *tox* is phenotypically silent, and even under conditions of optimal *tox* expression C7(γ *tox*) fails to produce detectable levels of toxin-related polypeptides. We have shown, however, that the insertional inactivation of the γ phage *tox* allele does not inhibit transcription from the *tox* promoter. The *C. diphtheriae tox* transcript was detected in extracts of iron-limited C7(γ *tox*) by S1 nuclease mapping experiments; however, this transcript was not detected in extracts of cells grown in iron-supplemented medium. In addition, the 5′ terminus of the *tox* transcript was found to be identical to that of the transcript in C7(β *tox*$^+$) and PW8 strains of *C. diphtheriae*, as well as recombinant *E. coli*(pDT201). These results dem-

onstrate that the *tox* promoter in corynephage γ *tox* is functional and is regulated by iron in a manner analogous to that of the *tox* promoters of corynephages β tox^+ and ω tox^+. In particular, these results rule out the possibility that sequences beyond bp 54 of the *tox* coding region are required for the transcription or regulation of the *tox* gene.

ACKNOWLEDGMENTS

We thank Michel Kaczorek for sharing data on *tox* mRNA and Rino Rappuoli for sharing data on the DNA insertion in the corynephage γ *tox* prior to publication. We also thank Mary Betlach for editorial advice.

This research was supported by Public Health Service grant AI-21628 from the National Institute of Allergy and Infectious Diseases.

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